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Radiation Enhances Silica Translocation to the Pulmonary Interstitium and Increases Fibrosis in Mice

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The effects of whole body irradiation (WBR) on particle clearance and the development of pulmonary fibrosis have been investigated. Using carbon, clearance is accomplished by polymorphonuclear leukocytes (PMN) and alveolar macrophages (AM), and only a few particles reach the interstitum. However, in preirradiated mice, the usual eflux of inflammatory cells is much delayed so that more free carbon remains in the alveoli, and by 1 week, many particles cross the epithelium to be phagocytized by interstitial macrophages. Carbon is found in the peribronchiolar interstitium 6 months later with no evidence of fibrosis. In the present study, mice received 1 mg silica intratracheally 2 days after 6.5 Gy WBR when the white blood cell count was low. A much-reduced AM and PMN response was found in the following 2 weeks compared to the reaction to silica alone, and many silica particles reached interstitial macrophages. In this case, macrophage activation by silica was associated with fibroblast proliferation, and by 16 weeks, much more pulmonary fibrosis was produced than after silica or irradiation only. This was measured biochemically and correlated with a large increase in retained silica in the irradiation–silica group. The results indicate that radiation inhibits the inflammatory response to particle instillation, resulting in greater translocation of free particles to the pulmonary interstitium. In the case of silica, the greater, prolonged interaction with interstitial macrophages leads to a much exaggerated fibrotic reaction.

Introduction

Clearance of particles from the alveolar spaces is accomplished principallly by macrophages, which are subsequently eliminated by the mucociliary apparatus. The initial interaction of macrophages with particulate material releases a chemotactic factor(s) (*1*), so that the deposition of a large load of carbon, for example, is followed by a rapid outpouring of polymorphonuclear leukocytes (PMN) and new alveolar macrophages (AM), both of which phagocytize and clear particles (*2*). At high burdens, the number of AM produced appears finite: some free particles remain in the alveoli and may be translocated across the type I epithelium to the interstitial macrophages (*2*).

In kinetic studies of normal lung and after whole body irradiation, it was found that the AM population could be maintained by proliferation of macrophages in the interstitium with subsequent cell migration to the alveoli (*3,4*). In response to a particulate load, a dual origin of new AM was seen with local division being supplemented by rapid migration of monocyte-derived cells (*5*). For example, after carbon instillation to mouse lungs, the initial rise in alveolar macrophages at 1 day was not accompanied by a change in mitotic activity in the lung and could be accounted for by increased output and migration of monocytes from the marrow. In the second phase, after 1 day, macrophagic output was supplemented by local production in the pulmonary interstitium, with a small increase in division of free AM (*5*).

Since the adaptive increase in AM is essential to the clearance mechanism, it has been suggested that radiation could reduce particle clearance by eliminating the precursor cells in the marrow. This would reduce the eflux of monocytes and PMN to the alveoli in the early stages after particle deposition. We have previously shown that the instillation of carbon to preirradiated mice results in a lower-than-normal production of AM and enhanced particle translocation to the interstitium (*6*). Whereas retention of this inert particle was not followed by pathologic changes in the lung, it is possible that increased activation of interstitial macrophages by a particle such as silica could promote the fibrotic response.

Although most attention has been paid to the secretory activity of the alveolar macrophage in the generation of pulmonary fibrosis (*7,8*), there is a large population of macrophages in the pulmonary interstitium that is potentially more important because of its proximity to the fibroblast. We have now examined the development and extent of fibrosis in lungs exposed to silica in a situation where particulate clearance was depressed by whole-body irradiation (WBR), thereby allowing relatively free passage of particles to the interstitium. The cellular responses seen in the alveoli, the cytokinetics of various pulmonary cell types, and the development of fibrosis were correlated with the location and retention of silica in the lungs of the various groups.

Materials and Methods

Two groups of Swiss-Webster mice (25-g males, about 6 weeks old were exposed to 6.5 Gy WBR. The animals were housed in

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individual sections of a plastic box during exposure (*6*). Three days later, one of these groups received an intratracheal injection of 1 mg silica (mean diameter 0.3 μm; Dowson and Dobson, South Africa) in 0.1 mL sterile water while under mild nembutal anesthesia. A third group of mice was instilled with silica only, and a fourth group received no treatment and served as control, particularly for collagen levels. Animals in sets of four were killed at intervals to 16 weeks. Each mouse received 2 μCi(0.07 Bq)/g tritiated thymidine 1 hr before death.

The heart was punctured, 0.3 mL blood withdrawn, and the total white blood cell count (WBC) was determined using a Coulter counter. A tracheotomy was then performed and the lungs were washed four times with 1.0 mL saline. The lavage fluid was pooled for each animal, and the total number of cells was counted on a hemocytometer. The cell suspension was centrifuged and a cytospin preparation was made and stained. Differential counts of PMN and AM were made on 500 cells per slide, then the total number of cells of each type (mean ± SE for four mice) was calculated at each time.

After lavage, the bronchus leading to the right lung was clamped; this lung was removed, weighed, and frozen. Later the lung was homogenized in water, and a biochemical assay for hydroxyproline (HYP) as an index of collagen content was carried out after hydrolyzing the homogenate with hydrochloric acid (*9*). The left lung was inflated with 0.5 mL 2% buffered glutaraldehyde and removed; most of the tissue was processed for embedding in glycol methacrylate. Sections (0.75-μm thick) from three random blocks per animal were prepared for autoradiography using Kodak NTB2 emulsion. We determined the percentages of [^{3}H]thymidine-labeled cells at each time point by counting 3000 lung cells per animal. These sections were thin enough to allow identification of pulmonary cell types, and differential counts of labeled cells were perfomed on 300 labeled cells per animal. The product of the differential percentage for labeled interstitial cells and the total labeling percentage gave the labeling index for this cell type.

At 16 weeks, four extra mice from each group were killed, the lungs were removed, chopped up, and incubated in 40% KOH overnight in an 80°C water bath. When the tissue was completely digested, the solution was centrifuged at 1500 rpm for 15 min, and a residue was obtained. This was washed twice in distilled water, resuspended in water, and one drop placed on a copper grid for examination by electron microscopy. The remainder was dried and the weight of the residue was determined.

Results

Cell Quantitation

The WBC count from animals that received silica alone was not significantly changed from the control, noninjected mice. However, both groups that received irradiation showed a marked reduction in WBC in the following 2 weeks (Fig. 1). At the time when irradiated mice received silica, the WBC level was low, about 25% of the control. The leukocyte count decreased more, then recovered slowly and returned to the normal range by 4 weeks.

About 2 × 10^5 cells, all macrophages, were recovered from control mice by bronchoalveolar lavege, and the numbers were not altered in animals that received radiation only. In mice that received silica only, there was a rapid inflammatory response, and AM numbers rose 6-fold within a few days (Fig. 2). Although the large increase in AM declined over the next few weeks, the number of cells recovered remained above normal up to 12 weeks after silica exposure. When irradiated mice were instilled with particles, little effect on AM numbers was seen initially, then a 4-fold rise occured (Fig. 2). Although the values fell somewhat, the number of AM recovered remained above normal to 16 weeks.

The PMN response showed a similar pattern with a rapid rise in cells seen in the silica-only group, but when irradiated mice received silica, there was only a small increase in PMN in the lavage fluid over the first week, then a delayed increase to a peak at 2 weeks (data not shown). Although the number of PMN fell subsequently, some of these cells were found up to 16 weeks in lavage fluid from the silica-plus radiation group.

Morphology

Radiation Only. In the radiation-only group of mice there was ultrastructural evidence of endothelial cell injury in the first 2

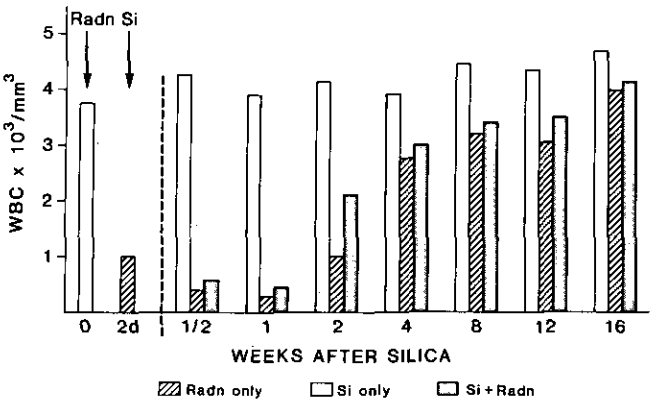

FIGURE 1. Numbers of white blood cells (WBC) in the radiation-only, silica-only, and combined treatment groups to 16 weeks. Control value is shown at time 0.

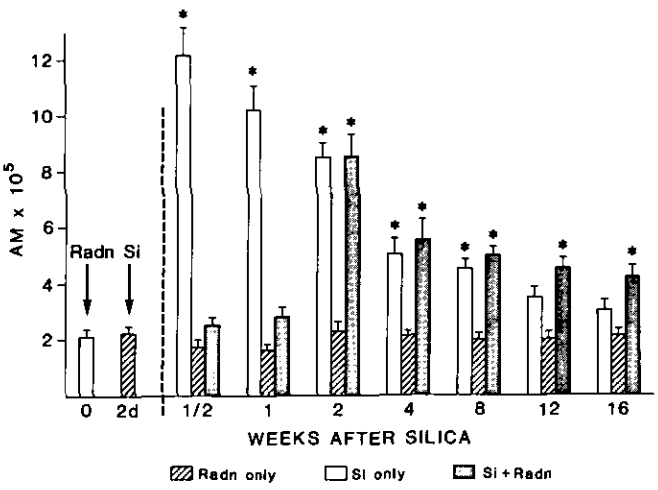

FIGURE 2. Numbers of alveolar macrophages (AM) in lung lavage after 1 mg silica, 6.5 Gy WBR, or both. (*) $p < 0.01$, value greater than control, time 0.

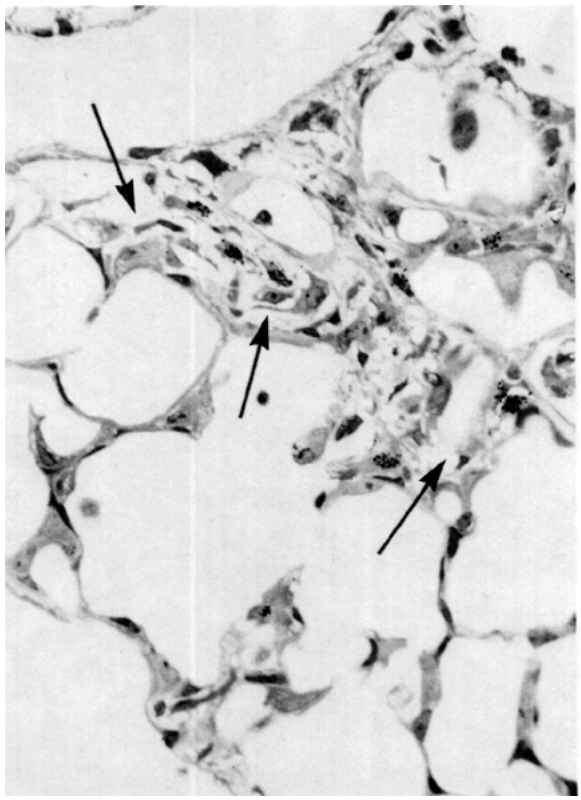

FIGURE 3. Lung section 12 weeks after whole-body irradiation only, showing alveolar walls thickened by fibrosis (arrows). × 750.

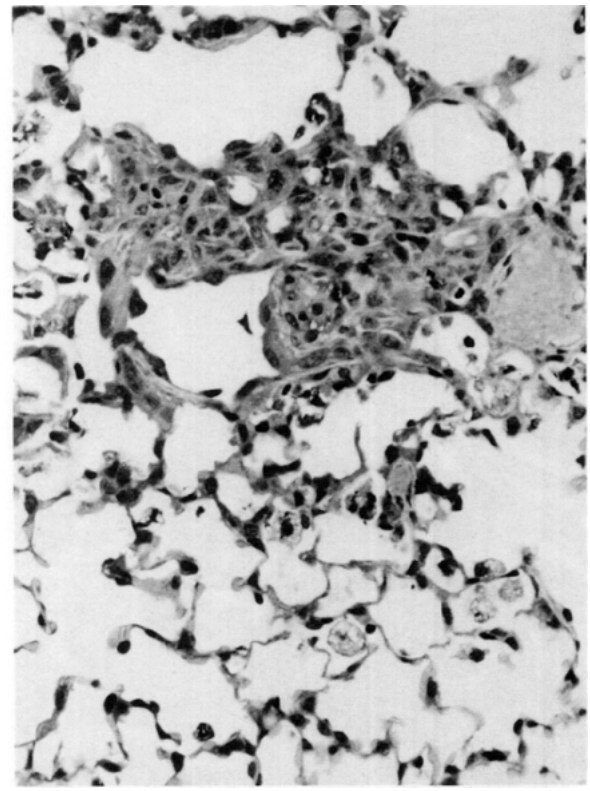

FIGURE 4. Lung section 8 weeks after silica only, showing a small granuloma with macrophages and fibroblasts. × 600.

weeks, but subsequently these cells appeared normal. By 4–8 weeks, focal thickening of some regions of the air-blood barrier by fibroblasts and collagen was seen, and linear scarring of alveolar walls was evident after that time (Fig. 3).

Silica Only. In the first 2 weeks, many PMN and AM were seen in the alveoli, and by electron microscopy, silica particles were found in both of these cell types. A few particles were observed at 3 days in type I alveolar epithelial cells and later in interstitial macrophages. There was some focal necrosis of type I cells at this time, followed by evidence of type II cell proliferation during repair. After 4 weeks, some small interstitial granulomas of macrophages and fibroblasts were seen (Fig. 4). These interstitial foci contained silica particles, and they became more fibrotic up to 16 weeks. In some peripheral alveolar regions there were large, free macrophages containing silica, often appearing trapped in the alveoli. These cells were seen up to 16 weeks, and the neighboring alveolar walls were thin, with few signs of fibrosis (Fig. 5).

Silica Plus Radiation. Up to 2 weeks after silica exposure, very few inflammatory cells were seen in the lung; the alveoli contained macrophages, some cell debris, and free silica. In contrast to the silica-alone group, particles were seen frequently within the type I cell cytoplasm, and by 2 weeks many interstitial macrophages contained phagocytized particles (Fig. 6). At this time, large granulomas of mixed macrophages and fibroblasts were evident in areas where ther was a heavy load of interstitial silica (Fig. 7). These interstitial granulomas became more fibrotic with time but still contained macrophages and silica particles.

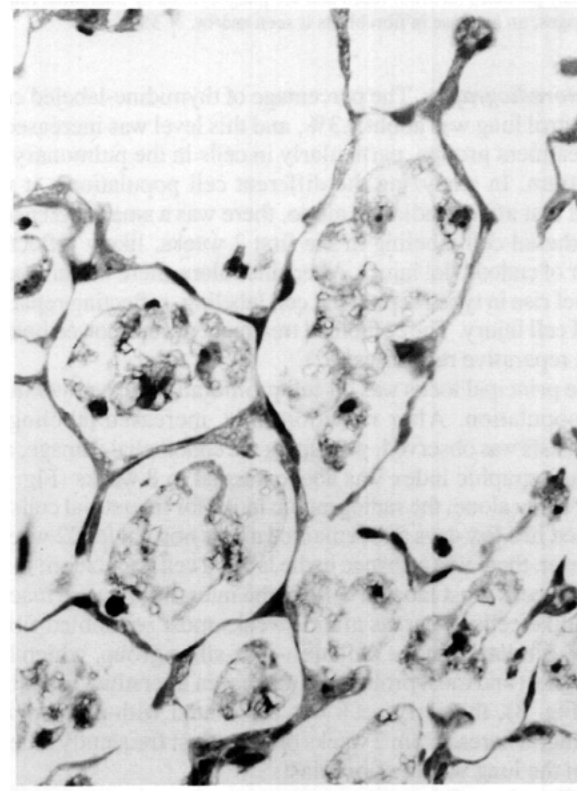

FIGURE 5. Lung section 12 weeks after silica only, showing a peripheral area where many alveolar macrophages contain particles. Alveolar walls nearby are not thickened. × 750.

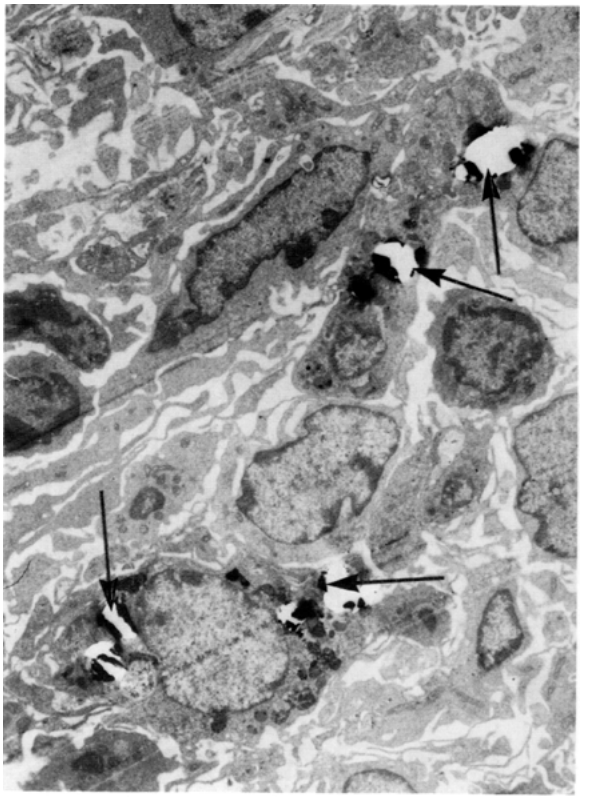

FIGURE 6. Electron micrograph of lung 4 weeks after radiation plus silica. Many particles (arrows) reach and are phagocytized by interstitial macrophages; an increase in fibroblasts is seen nearby. × 3500.

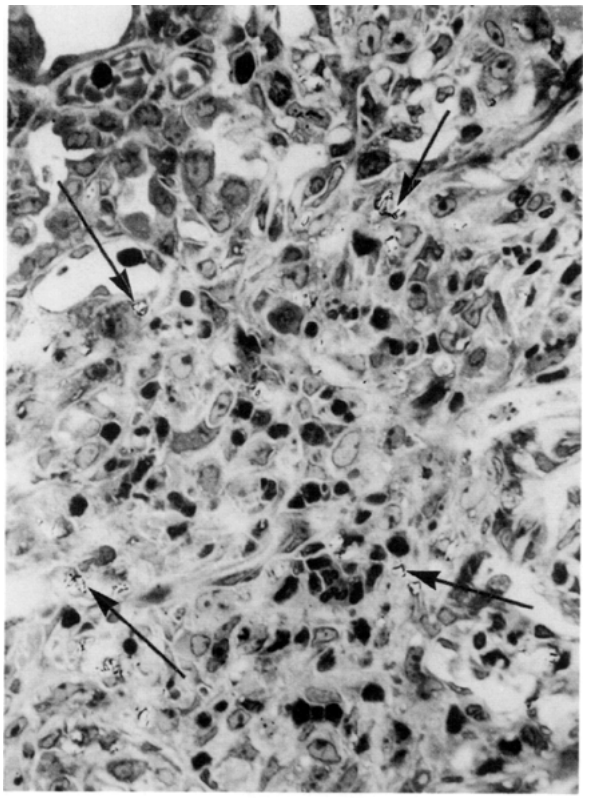

FIGURE 7. Lung section 12 weeks after radiation plus silica. A large interstitial granuloma with fibroblasts and macrophages is seen. Many silica particles (arrows) are located in this lesion. × 600.

Autoradiography. The percentage of thymidine-labeled cells in control lung was about 0.3%, and this level was increased in all treatment groups, particularly in cells in the pulmonary interstitium. In analyzing the different cell populations, it was found that after irradiaiton alone, there was a small increase in endothelial cell labeling in the first 2 weeks, likely reflecting repair of endothelial injury. After silica alone there was an equally brief rise in type II epithelial cell labeling, reflecting repair of type I cell injury. The combined treatment group showed both of these reparative responses (*10*).

The principal focus was on cell proliferation in the intersitital cell population. After radiation only, increased labeling of fibroblasts was observed, postdating the endothelial damage, and the radiographic index was above normal to 8 weeks (Fig. 8). After silica alone, the radiographic index for interstitial cells increased in a few days and remained above normal for 12 weeks. However, there was a change in the labeled cell population; in the first 2 weeks most labeled cells in the interstitium were macrophagelike cells, whereas after 2 weeks most resembled fibroblasts. Similarly, in the radiation-plus-silica group, which had the highest and most prolonged increase in interstitial cell labeling (Fig. 8), the early peak was associated with macrophage labeling, whereas from 2 weeks on, the most frequently labeled cell in the lung was the fibroblast.

Chemistry. To quantitate pulmonary fibrosis, the total HYP content of the right lung was measured. Irradiation or silica exposure alone resulted in increased HYP beginning at 4 weeks to

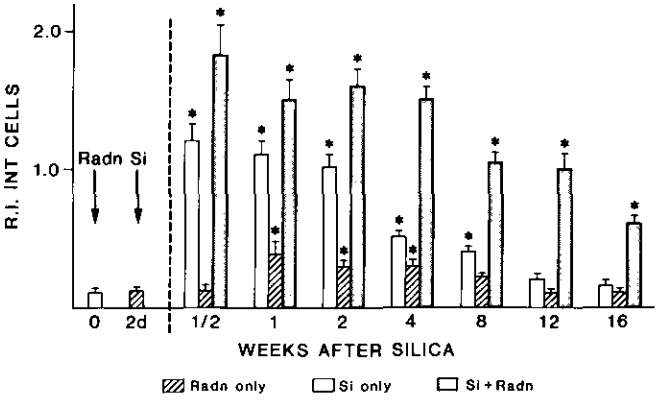

FIGURE 8. Radiographic index (RI) of interstitial cells (INT) in the various groups. (*) $p < 0.01$ greater than control at time 0.

give a 50% increase at 16 weeks (Fig. 9). The combination of irradiation followed by silica injection produced an increase in HYP by 2 weeks, and the level continued to rise to 16 weeks when the level was more than two times control values and larger than all other groups. The degree of fibrosis was also greater than that predicted if the value obtained for silica alone or radiation alone were added together (Fig. 9).

To relate the fibrotic response to the retention of silica, the weights of residues recovered after tissue digestion were meas-

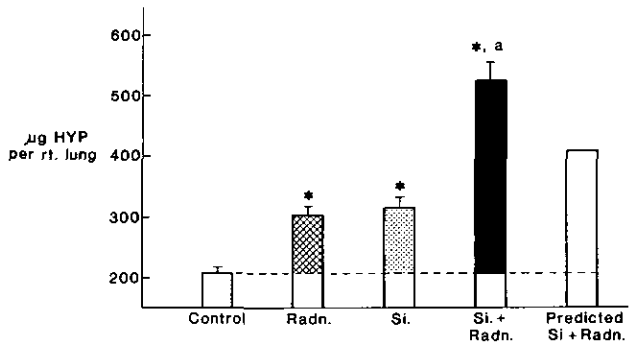

FIGURE 9. Hydroxyproline (HYP) content of right lung 16 weeks after radiation, silica, or combined treatment. (*) Vaues > control; (a) value > all other groups.

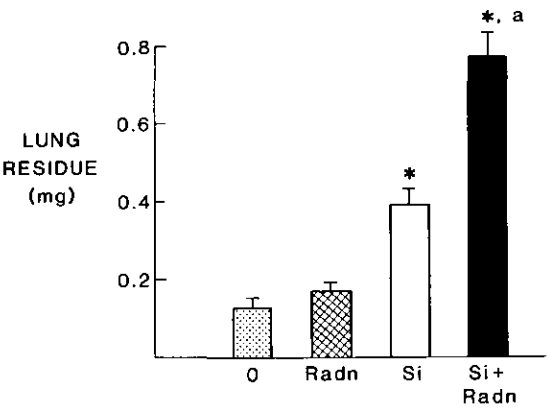

FIGURE 10. Weight of lung residue recovered from each group at 16 weeks. (*) Values > control; (a) value > all other groups.

ured (Fig. 10). An amorphous, nonparticulate residue was obtained from normal lung and from the fibrotic lung after irradiation alone. The residue from mouse lung after instillation of silica was whitish in appearance, contained particles as seen by electron microscopy, and weighed twice as much as that from controls. After digesting tissue from the irradiation-plus-silica group, a white residue, rich in silica, was obtained, and this residue weighed significantly more than that prepared from other groups.

Discussion

The initial cellular response to particle instillation into the lung is granulocytic, and large numbers of PMN rapidly cross both alveolar and bronchiolar epithelia. Initially, PMN are recovered in larger numbers than macrophages, and they also phagocytize and clear particles. The PMN response, however, is usually short lived, and levels drop within 1 week even after a heavy load (*2,10*). Over the longer term, particle elimination requires an adaptive increase in the number of macrophages. Various studies on the origin of the AM indicate that in normal animals, the population is maintained by local proliferation (*3,4*). This may occur in AM *in situ* (*11*) or in precursor cells in the interstitium (*3,12*), and several studies that indicate a local origin of AMs refer to pulmonary macrophage proliferation without distinguishing cellular location (*13,14*). In the present study, there was no reduction in AM in mice receiving radiation only, further supporting a local origin for these cells in monocyte-depleted animals.

In response to particulate loading, a dual origin for the adaptive increase in AM has been shown (*2,5*). The number of macrophages increases at 1 day due to cellular migration, whereby monocytes cross from blood to alveoli. After day 1, however, the continued macrophagic output is related predominantly to increased division by pulmonary interstitial cells. When whole-body irradiation is used to eliminate the early PMN response and the migration of blood monocytes following particle instillation, the subsequent AM count is lower than that obtained after silica alone and does not increase until 1–2 weeks after particle deposition. The principal consequence of a delayed or reduced adaptive response of the macrophagic system is reduced particle clearance and enhanced translocation to the interstitium. In general, a few inhaled or instilled particles have been observed crossing the cytoplasm of type I alveolar epithelial cells before phagocytosis by interstital macrophages (*2,15,16*). These interstitial phagocytes may remain embedded in connective tissue for a long time or may migrate to the alveoli or lymphatics (Fig. 11). Deposition of high numbers of particles in the alveoli increases the chance of contact with type I epithelial cells and passage into the lung tissue to reach interstitial macrophages. This effect has been seen in a conventional overload situation (*2*) and in phagocyte-depletion experiments where irradiated mice received carbon or silica (*5,10*). In each case, a large increase in particle retention occurs in peribronchiolar interstitial macrophages.

In the present study, instillation of silica to the lungs of irradiated mice resulted in a large increase in translocation of

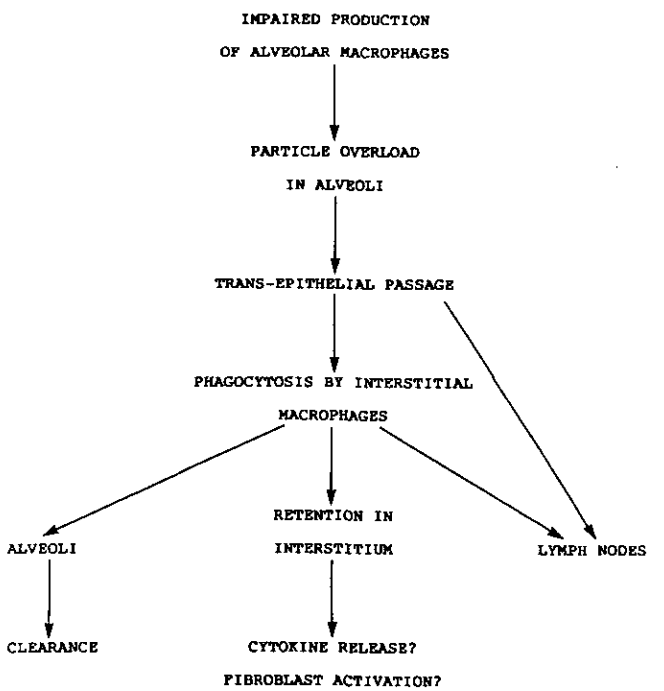

FIGURE 11. Pulmonary reactions to instilled particles when the AM response is reduced.

silica to the interstitium. Many particles were found in interstitial macrophages over the 16-week period, in association with fibroblasts within collagen-forming granulomas. In regions of the lung where silica was confined to free AM within the air sacs, there was no obvious thickening of alveolar walls. After 16 weeks when the lung tissue was digested, twice as much residue, mainly silica, was recovered from the irradiation-plus-silica group, and the prolonged retention of silica mostly in the interstitial macrophages over 16 weeks was associated with a significant increase in fibroblastic proliferation and collagen deposition. Thus, the enhanced translocation of silica to interstitial macrophages resulted in greater pulmonary fibrosis than occurred when the same dose of silica was handled predominantly by the AM within the air sacs.

The type of granulomatous fibrosis seen after combined irradiation plus silica is clearly an exaggeration of the pulmonary response to silica. The predominant dividing cell in the lung after 2 weeks was the fibroblast, and the ongoing high level fibroblast proliferation and collagen deposition seen in the silica-plus-irradiation group likely reflects secretion of a fibroblast growth factor by particle-laden interstitial macrophages trapped within the granulomas. The role of macrophage secretions in fibrogenesis is well established (*7*), but most studies of the lung have concentrated on secretory activity of the free AM (*8*). The pulmonary interstitial macrophage is potentially more important because of its proximity to the fibroblast. In addition, alveolar and interstitial macrophages are different (*17*), and they may not contribute equally to the fibrogenic process. Whereas secretion of a macrophage-derived factor into the alveolar space may not reach the interstitial fibroblast due to inactivation or poor passage across the epithelium, any macrophage-derived factor generated in the interstitium may be communicated directly to adjacent fibroblasts. During overload, the high level of particle phagocytosis by the interstitial macrophage and its subsequent activation could permit such direct transfer of any growth factors to the fibroblast. Thus, it may be inferred that any pulmonary condition that results in a diminished inflammatory response, or in decreased phagocytosis, will result in an excess of free particles in the alveoli and so will increase the likelihood of transepithelial passage of particulates to reach the interstitial macrophage. Particle-induced changes in these macrophages by an agent such as silica, and any resulting macrophage–fibroblast interaction at this location, is more likely to result in structural and functional changes in the lung (Fig. 11).

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